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Nucleic acids which code for nuclear base transporters

The present invention concerns a nucleic acid which codes for a plant or animal nuclear base transporter, and its use. In addition the present invention concerns a fragment of the nucleic acid, a construct containing the nucleic acid and/or a fragment of it, and a host cell. Included in addition in the present invention is a process for the manufacture of a transgenic cell as well as a process which influences the nuclear base transporter properties of a plant, a part of a plant and/or of seeds.

Transporters play a particular role in the functioning of an organism. On the one hand they determine the uptake or emission of a substance into or out of a cell or an organism, on the other they control the transport and distribution of substances between the cells. As a rule transporters lie at the beginning or the end of a metabolic pathway and thereby take charge of fundamental higher controlling functions.

Purine and pyrimidine bases and the nucleosides and nucleotides derived from them are numbered among the transporter metabolites which serve as the building blocks of the nucleic acids. The uptake of these substances, for example during pollen fertilization and the early development of the embryo in germinating seeds, has an important physiological significance in the preparation of early stages for the synthesis of nucleic acids. As phytohormones, cytokinins are structurally closely related to the purine bases and the purine nucleosides. These phytohormones regulate many processes during plant development. Very little is known about the origin of these hormones, yet their effective transport in the plant is of decisive importance.

In bacteria nuclear base transport systems for adenine, cytosine and uracil were characterized and the corresponding genes could be cloned. In the baker's yeast *Saccharomyces cerevisiae* three differing active transport systems for nucleosides and nuclear bases have so far been well characterized both genetically and physiologically. Nucleoside transport systems have been described and characterized in a number of mammalian cells. Besides these nucleoside transporter systems specific transporter systems for nuclear bases have also been described.

In higher plants transport processes for distribution of assimilates, metabolites and phytohormones are of critical physiological importance. Only very little is yet known about the transport of nuclear bases and their derivatives in plants, and up till now in contradistinction to bacteria, fungi and mammals only a few transport systems for these substances have been described. Clarification of the course of events of nuclear base transport in plants, giving similarly detailed information as that put in place for other organisms, is not available and owing to the difficulty of molecular biological analysis in plants, is scarcely practicable. Nevertheless, there exists great interest in the identification and characterization of plant genes coding for nuclear base transporters or transporters for chemically related substances. In addition on account of the central function of transporters a great interest exists in plants, which are in a position to transport great quantities of nuclear bases and their derivatives, as well as in the provision of possibilities for altering the distribution of nuclear bases in transgenic plants and mutants.

The present invention is based on the problem of providing nucleic acids coding for plant or animal nuclear base transporters.

This problem is solved in conformity with invention through a nucleic acid, chosen from:

a) nucleic acids obtainable through complementing nuclear base transporter-deficient host cells with a plant or animal gene bank and selecting nuclear base transporter-positive host cells;

b) nucleic acids with a sequence coding for a protein with a sequence following SEQ ID NO 8 or SEQ ID NO 9;

c) nucleic acids hybridizing with a nucleic acid according to b);

d) nucleic acids which, in view of the degeneration of the genetic code, would hybridize with a nucleic acid according to b) or with the sequence complementary to b)

e) through substitution, addition, inversion and/or deletion of one or more bases, obtained derivatives of a nucleic acid according to a) to d);

f) nucleic acids complementary to a nucleic acid according to one of groups a) to e);

excluding nucleic acids with a sequence according to one of the SEQ ID NO 3 to 5

The concept "nuclear base" as used here includes not only nuclear bases and their derivatives but also substances chemically related to the nuclear bases, for example adenine, guanine and their derivatives such as xanthine, hypoxanthine, allantoin, allantoate, urate, xanthosine or inosine, cytosine and its antecedent stages and derivatives such as barbiturates or folic acid, cytokinines, such as for instance zeatine, isopentenyladenine or kinetine, and certain alkaloids, such as for instance caffeine, theobromine or nicotine. These plant-derived alkaloids show a great structural similarity to the nuclear base purine, since they likewise embody basic N-containing heterocycles. Cytokinines contain adenine as hydrophilic basic structure, on whose amino-group in position 6 a non-polar side-chain of relatively limited specificity is situated. With kinetine what is concerned is an artificial cytokinine which apparently does not occur naturally at all in plants. Furthermore the nuclear bases can be modified and bound to sugar or other building-blocks, e.g. adenosine as riboside of adenine, cytidine as riboside of cytosine, or cytokinine ribosides.

The term "nuclear base transporter" in the sense of the invention signifies a protein which takes part in the transport of at least one of the aforenamed metabolites through a biomembrane. This transport may happen either actively or passively. For the indication of transporter activity the method described by Ninnemann et al. (1994, *EMBO J.* 15, 3464-3471) for example may be used.

"Complementation" as used here means a compensation in the phenotype of a genetic functional defect by maintenance of the mutation on which the defect is based.

Complementation in the sense of the invention is present when, for instance, a genetic defect in a gene (e.g. the FCY2 gene in *Saccharomyces cerevisiae*) is abolished by the presence of a similar gene (e.g. the PUP1 gene from *Arabidopsis thaliana*) which takes over the function of the defective gene.

Under "nuclear base transporter-deficient host cells" in the sense of the invention one understands cells which on account of a genetic defect display negatively altered nuclear base transporter properties which lead to a negatively selectable phenotype. In carrying out the invention the preferred nuclear base transporter deficient host cells are eukaryotic cells, e.g. plant or animal cells. Nuclear base transporter deficient yeast cells are especially preferred.

Nuclear base transporter positive host cells contain a nucleic acid which leads to at least partial abolition of the genetic defect and hence manifest a positively selectable phenotype.

The identification of a nuclear base transporter may for example result through complementation of specific mutations in the yeast *Saccharomyces cerevisiae*. For isolation of a gene coding for a transporter molecule, the most closely suitable yeast mutants from a plant or animal gene bank must be available, which on account of a defect in this transporter molecule are not in a position to take up a specific substance. A mutant which is not in a position to grow in media with nuclear bases as the only source of nitrogen is for example the *fcy2* mutant described by Grenson (strain MG887) (Grenson , 1969, *Eur. J. Biochem.* 11, 249-260; Polak and Grenson, 1973, *Eur. J. Biochem.* 32, 276-282). In order to be able to carry out complementation with plant or animal genes, the URA3 gene was destroyed and a uracil auxotrophe thereby produced (MG887ura3$^-$).

For obtaining a nucleic acid according to the invention, a suitable yeast mutation, such as for example the *fcy2/ura3* mutant, with expression plasmids suitable for use in yeast, which it carries as insertion cDNA fragments from a plant or animal cDNA library, can be transformed. Plant or animal nuclear base transporters are identified through selection of transformants which as a result of the expression of plant or animal cDNA sequences are in a position to grow on nuclear bases as the only source of nitrogen,.

Surprisingly, it is now found that with expression of a cDNA library, for example from germinal tissue of *Arabidopsis thaliana*, by means of the use of suitable expression plasmids in yeast containing the phosphoglycerate kinase promotor from yeast, complementation of the *fcy2* mutation is possible if the expression plasmids contain specified plant cDNA fragments. These cDNA fragments code for plant nuclear base transporters and are included in the present invention.

The expression "nucleic acids which hybridize with a nucleic acid according to b)" as used here indicates a nucleic acid which under extremely stringent conditions hybridizes with the nucleic acid according to b). For example, the hybridization with the radioactive gene specimen in a hybridization solution (25% formamide; 5 x SSPE; 0.1%SDS; 5 X Denhardt solution; 50µg herring-sperm DNA; for the composition of individual components compare Sambrook et al., 1989, Molecular Cloning: a

laboratory manual, 2nd edn., Cold Spring Harbour Laboratory Press, NY, USA) can result in 20 hours at 37°. The concluding removal of non-specifically bound specimens can be carried out through multiple washing of the filter in 2 x SSC/ 0,1% SDS at 42°. Preferably the filter is washed with 0.5 x SSC/ 0.1%SDS, especially preferably with 0.1 x SSC/ 0.1%SDS at 42°.

The nucleic acids according to the invention can be introduced into plasmids and by means of standard microbiological procedures undergo mutagenesis or a sequence change through recombination. In this way a particularly simple alteration in specificity of the nuclear base transporter is possible. Nucleic acids which code for changed nuclear base transporters can for example be used for the transformation of agriculturally cultivated plants with the aim of producing transgenic plants. With assistance from standard procedures (cf. Sambrook et al., 1989, Molecular cloning: a laboratory manual, 2nd edn., Cold Spring Harbour Laboratory Press, NY, USA) base replacement can be undertaken or natural or synthetic sequences added. For the bonding of fragments to one another adaptors or "linkers" may be added to the fragments. In addition manipulations which provide appropriate cleavage sites or remove superfluous sequences or cleavage sites may be inserted. In order to undertake insertions, deletions or substitutions, such as e.g. transitions or transversions, one makes use of recognized methods such as eg. *in vitro* mutagenesis, "primer repair", restriction or ligation. For analysing the nucleic acids associated with the invention ordinary methods such as e.g. sequence or restriction analysis are used, as well as further biochemical and molecular biological methods.

A nucleic acid coding for a polypeptide or protein with nuclear base transporter activity, which over its whole sequence displays at least 40%, preferably at least 60%, 80% being especially preferred, homology with a polypeptide coded by the nucleic acid according to SEQ ID NO 1 or the nucleic acid according to SEQ ID NO 10, is similarly included in the present invention.

In the context of the invention the expression "at least 40%, preferably at least 60%, 80% being especially preferred, homology" is related to agreement at the level of the amino acid sequence which can be determined according to recognized procedures, e.g. computer-supported sequence comparison (Basic local alignment research tool, S. F. Altschul et al. J. Mol. Biol. 215 (1990), 403-410).

The expression "homology", known to the specialist, signifies the degree of relationship between two or more polypeptide molecules determined through the agreement between the sequences, by which under "agreement" both identical agreement and conservative amino-acid replacement are to be understood. The percentage amount of "homology" is derived from the percentage portion of regions agreeing in two or more sequences bearing in mind gaps and other sequence peculiarities.

The expression "conservative amino-acid replacement" refers to a replacement of an amino acid residue with another amino acid residue by which the replacement does not lead to an alteration of the polarity or charge. An example of a conservative amino-acid replacement is the replacement of a non-polar amino-acid residue by another non-polar amino-acid residue. Conservative amino-acid replacements in the

sense of this invention are: G = A = S, I = V = L = M, D = E, N = Q, K = R, Y = F, S = T, G = A = I = V = L = M = Y = F = W = P = S = T.

The homology of polypeptide molecules related to one another can be determined with the help of known procedures. As a rule special computer programmes have the calculation-containing algorithms for particular requirements installed. Preferred procedures for the determination of homology produce at the start the greatest agreement between the sequences investigated. Computer programmes for the determination of the homology between two sequences contain but are not limited to the GCG programme package, including GAP (Devereux, J., et al., Nucleic Acids Research 12 (12): 387 (1984); Genetics Computer Group University of Wisconsin , Madison , (WI)); BLASTP, BLASTN and FASTA (Altschul, S. et al., J. Molec. Biol. 215: 403/410 (1990)). The BLASTX programme can be obtained from the National Centre for Biotechnology Information (NCBI) and from further sources (BLAST Handbook, Altschul S. et al., NCB NLM NIH Bethesda MD 20894; Altschul, S., et al., J. Mol. 215: 403/410 (1990)). The well-known Smith Waterman algorithm may also be used for the determination of homology.

Preferred parameters for the comparison of sequences include those below:

Algorithm:	Needleman and Wunsch, J. Mol. Biol 48: 443-453 (1970)
Comparison matrix:	BLOSUM 62 by Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89: 10915-10919 (1992)
Gap penalty:	12
Gap length penalty:	4
Threshold penalty of similarity	0

The GAP programme is also suitable for use with the above parameters. The above parameters are the standard parameters (default parameters) for amino-acid sequence comparisons.

Further specimen algorithms, gap opening penalties, gap extension penalties, comparison matrices including the Wisconsin package version 9 , September 1997, mentioned in the programme handbook, may be used. The choice depends on the comparison being carried out and furthermore on whether the comparison is being carried out between pairs of sequences, for which GAP or Best Fit are preferred, or between a sequence and a comprehensive sequence data bank, for which FASTA or BLAST are preferred.

A nucleic acid which codes for a polypeptide or protein with nuclear base transporter activity, which over a section of at least 20 amino-acids displays at least 60%, preferably at least 75%, 90% being especially preferred, homology with one of the polypeptides coded for by the nucleic acids according to SEQ ID NO 1 and the nucleic acids according to SEQ ID NO 10, is similarly included in the invention. Preferably the nucleic acid sequence according to the invention codes for a polypeptide or protein with nuclear base transporter activity which includes a nucleic acid sequence which displays at least 70%, preferably at least 80%, 90% being especially preferred, homology with one of the following amino-acid sequences:

(a) L Y A X G L X Y L P V S T X S L I X X X Q L A F X A X F S (SEQ ID NO 11)

in which X in positions 4 and 7 stands for a hydrophobic amino-acid (G, A, I, V, L, M, Y, F, W, P, S, or T), in positions 14 and 18-20 for an optional amino-acid, at position 25 for T or N and at position 27 for I or F;

(b) L G X V G L I F X X S S L F S X V X X X X X L P V (SEQ ID NO 12)

in which X at positions 3 and 18-22 stands for a hydrophobic amino-acid (G, A, I, V, L, M, Y, F, W, P, S or T), at position 10 for an optional amino-acid, at position 9 for L or E and at position 16 for G or N;

(c) L L L X I W G F X S Y X Y X (SEQ ID NO 13)

in which X in positions 9 and 12 stands for a hydrophobic amino-acid (G, A, I, V, L, M, Y, F, W, P, S or T), in position 4 for S or A and in position 14 for Q or S.

The nucleic acids preferably contain the coding sequence for one of the sequences according to the SEQ ID NO 1, 2, 6, 7, or 10, or one of these through substitution, addition, inversion and/or deletion of one or more bases obtained derivatives. In a particularly preferred model of the invention the nucleic acid is a DNA.

The subject of the invention is similarly a fragment of the nucleic acid according to the invention, which in anti-sense orientation to a promoter can limit the expression of a nuclear base transporter in a host cell. This fragment can contain at least 10, preferably at least 50 , very particularly preferably at least 200 nucleotides. This fragment can be introduced into a host cell and there transcribed into a non-translatable RNA (anti-sense RNA) which through bonding to an endogenous nuclear base transporter gene or to the mRNA transcribed therefrom can inhibit their expression.

The invention further concerns a construct which contains a nucleic acid according to the invention and/or a fragment according to the invention, itself under the control of the expression-regulating elements. Examples of such regulating elements are constitutive or inducible promoters, such as for bacteria the *E.coli* promoter araBAD (Carra & Schlief, 1993, *EMBO J.* 12, 36-44), for fungi the yeast promoter PMA 1 (Rentsch et al., 1995, *FEBS Lett.* 370, 264-268) and for plants the viral promoter CaMV35S (Pietrzak et al, *Nucl. Acids Res.* 14, 5857-5868). In addition the nucleic acids or the fragment can be provided with a transcription termination signal. Elements of that kind have already been described (see e.g. Gielen et al, 1984, *EMBO J.* 8, 23-29). The transcription initiating region may be either native (homologous) or foreign (heterologous) to the host organism. The sequence of the transcription initiating and termination regions may be artificially manufactured or naturally acquired or contain a mixture of synthetic and natural components. Preferably the nucleic acid or the fragment in the construct are found in anti-sense orientation to the regulator element. The construct can for example be introduced into the plant genome and after its transcription lead to suppression of the formation of nuclear base

transporter molecules suitable for plants. In an especially preferred embodiment of the invention the construct is present in a plasmid.

The plasmid can contain a replication signal for *E. coli* or yeast and a marker gene, permitting positive selection of host cells transformed with the plasmid. If the plasmid is introduced into a plant host cell, further sequences known to the expert may be required, each depending on its mode of introduction. If the plasmid according to the invention is for example a derivative of the Ti or Ri plasmid, the introducing nucleic acid or the introducing fragment must be flanked by T-DNA sequences which facilitate integration of the nucleic acid or the fragment into the plant genome. The use of T-DNA for the transformation of plant cells has been intensively investigated. and is described among others in EP 120 516, Hoekema, The Binary Plant Vector System, Offset-printer Kanters B.V. Ablasserdam (1985), chapter 5, Fraley et al *Crit. Rev. Plant Sci.* 4, 1-46 and An et al., 1985, *EMBO J.* 4, 277-287. Once the introduced nucleic acid or fragment is integrated into the genome, it is as a rule stable therein and is contained also in the progeny of the original transformed cell. The integrated sequence may similarly contain a selection marker which confers resistance to a biocide or an antibiotic such as kanamycin, G418, bleomycin, hygromycin, or phosphinotricin on the transformed cell. The marker individually used should thereby distinguish cells transformed by selection from cells in which the introduced DNA is lacking.

The subject of the invention is in addition a host cell containing a nucleic acid according to the invention and/or a nucleic acid with a sequence according to one of the SEQ ID NO 3 to 5 and/or a fragment of the aforenamed nucleic acid and/or a construct according to the invention. The host cell in conformity with the present invention may be chosen from bacteria, yeast, mammalian and plant cells.

In addition the present invention concerns a transgenic plant as well as plant cells and/or seeds of this plant, containing a nucleic acid according to the invention or a nucleic acid with a sequence according to one of the SEQ ID NO 3 to 5 and/or a fragment of the abovenamed nucleic acids and/or a construct according to the invention. Preferably the nucleic acid, the fragment and/or the construct integrates into a site on the genome which does not correspond to its natural position.

The present invention similarly concerns a protein which through expression of a nucleic acid according to the invention or a nucleic acid with a sequence according to one of the SEQ ID NO 3 to 5 is obtainable in a host cell. The relevant initiation codons (ATG) of nos. 1 to 7 have been identified in the sequence listing underlining. Preferably the protein possesses the same nuclear base transporting properties as those of the protein coded by the SEQ ID NO 1 or the SEQ ID NO 9. As indication of the activity of such a protein uptake experiments may be carried out, as described in the examples. Antibodies which react with a protein according to the invention are similarly included in the invention.

A further object of the invention is the provision of a procedure for the production of a transgenic plant. This object is accomplished through a procedure consisting of the following steps:

- Introduction of a nucleic acid according to the invention or a nucleic acid with a sequence according to one of the SEQ ID NO 3 to 5 and/or a fragment, conforming to the invention, of the aforementioned nucleic acid, into a plant cell; and

- Regeneration of a plant from the transformed plant cell.

Transgenic plants which are produced in accordance with the invention procedure may be derived from e.g. tobacco, potato, sugar-beet, soya-bean, coffee, pea, bean, cotton, rice or maize plants.

For the introduction of the nucleic acid or the fragment into a plant cell there are besides transformation with the help of agrobacteria still numerous further techniques available. These techniques include the fusion of protoplasts, the microinjection of DNA, electroporation such as projectile methods and virus infection.
From the transformed plant cells entire plants can then be regenerated in a suitable medium, which may contain antibiotics or biocides for selection. Plants obtained in this way can then be tested for the presence of the introduced DNA.

Unlike in transformation with the assistance of agrobacteria no special demands are made on the vector by injection and electroporation. Simple plasmids such as pUC derivatives can be used. Should whole plants be regenerated from cells transformed by such means, however, the presence of a selectable marker gene is advantageous. The transformed cells grow inside the plants in the usual way (see also McCormick et al., 1986, *Plant Cell Reports* 5, 81-84). These plants can as usual be raised and crossed with plants possessing the same transformed or other genetic background. The hybrid individuals resulting therefrom have the corresponding phenotypic properties.

The subject of the present invention is similarly a procedure for influencing the nuclear base transporter properties of a plant, part of a plant, a plant cell, and/or of seeds, which contains the following step:

- Introduction of a nucleic acid in conformity with the invention or a nucleic acid with a sequence according to one of the SEQ ID NO 3 to 5 and/or a fragment, in conformity with the invention, of the aforementioned nucleic acid, into a plant cell or a plant.

For influencing the nuclear base transporter properties of a plant, both alterations in the specificity of the transport system which facilitate the transport of new combinations, and those which evoke a change in the transport mechanism, are appropriate. For example alterations are possible which change the affinity or substrate specificity of the transporters resulting in a more efficient nuclear base transport in the leaves or changes in apical dominance, the process of blooming or senescence, or make possible an improved dispersion of pesticides.

The plant cells in accordance with the invention can be used for the regeneration and production of entire plants. The nucleic acids in accordance with the invention as well as nucleic acids with a sequence according to one of the SEQ ID NO 3 to 5 can be used for isolating homologous sequences from bacteria, fungi, plants, animals and/or people. To be able to search for homologous sequences, gene banks must first be

established which are representative of the gene profile of an organism or of the expression of genes in this organism. Firstly there are genomic, lastly there are cDNA banks. From these related sequences can be isolated with the help of a probe among the aforementioned nucleic acids. Once one has identified and isolated the gene belonging to it, determination of the sequence and an analysis of the properties of the proteins coded by this sequence is possible.

A further use of the aforementioned nucleic acids concerns the expression of a nuclear base transporter in prokaryotic and eukaryotic cells. If the aforementioned nucleic acids are introduced into a prokaryotic cell an RNA sequence of a eukaryotic nuclear base transporter translatable by bacteria is constructed, which despite the considerable differences in the membrane structure of prokaryotes and eucaryotes is translated into a functional eukaryotic nuclear base transporter with its substrate specificity. This makes possible the use of bacterial lines for studies of the transporter as well as its substrate. The aforementioned nucleic acids can similarly be used under the control of a regulatory element in anti-sense orientation for the inhibition of the expression of an endogenous nuclear base transporter in prokaryotic and/or eukaryotic cells. The manufacture of transgenic useful plants represents a further possibility for the use of these nucleic acids.

Further uses are:

- If the transporter is essential for the function of the plant, it can serve as a herbicide target: screening procedures in yeast in order to search for inhibitors, these can be optimized in the yeast system and through chemical modification and then tested on the plants.

- Since substitution in the basic structure of the substrate is allowed, one can use the transporter to mobilize pesticides, e.g. through affixing purine and pyrimidine residues to fungicide or insecticide.

Special uses are:

1. Substrate class A: Transporters are responsible for the transport of secondary metabolites or alkaloids such as caffeine, theobromine, nicotine and similar substances.

- Over-expression or ectopic expression under the control of various promoters such as CaMV-35S or specific promoters in order the better to transport secondary metabolites of these substance classes in particular organs, e.g. leaves at harvest-time, seeds and tubers or beetroots.

- Cosuppression or antisense repression in order to diminish the content of secondary metabolites of that type, especially toxic substances in particular organs, egg the products of nutrition; egg as a new type of decaffeinating agent.

- Secondary metabolites are important defensive substances of plants against infections and animal consumption. Improved care of the organs affected can lead to improved resistance and ability to fight back.

- Secondary metabolites as pharmaceutical products (plant as bioreactor). An optimal care of crop organs is essential for the extractability and can be attained by optimized transport in transgenic plants.

2. Substrate class B: Transporters are responsible for the transport of nuclear bases and derivatives, e.g. on the one hand adenine, xanthine, allantoin, hypoxanthine, urate, xanthosine, inosine; on the other hand cytosine and its derivatives such as barbiturate and folic acid, but also nucleosides such as adenosine, etc. Reversal of transport processes in transgenic plants leads to

- altered cell division activity and improved development of autonomous cells: nuclear bases play an important role in DNA and RNA synthesis and thereby in cell division activity, for example pollen is supplied with nuclear bases. Useful for the creation of sterile male pollen through transport inhibition.

- in the transport of allantoic acid a role in the fixation of atmospheric nitrogen. Intercellular transport is important for the nitrogen assimilation of plants.

- Adenine is an essential building-block for ATP. The external supply e.g. of the phloem with ATP could, through alteration of expression, be influenced positively or negatively in transgenic plants by PuP transporters.

3. Substrate class C: Transporters are responsible for the transport of plant hormones, e.g. cytokinins and cytokinin derivatives, e.g. ribosides (see adenine/adenosine).

Since cytokinins play a central role in the control of development and metabolic processes, an alteration in activity can, due to

- Over-expression, ectopic expression or repression (through cosuppression or anti-sense) in transgenic plants lead to improved endurance (control of sink-source relationships), changed degree of branching (role in apical dominance), improvement in the germination situation of seeds, delay or prolongation of bud formation and delay or prolongation of senescence (cytokinins prolong senescence). In addition the cell division activity is influenced, and thereby the formation of side-roots, the size and capacity of the organs of production, morphogenesis in tissue culture, the expansion of the leaves and the regulation of water efficiency on account of the influence on stoma opening and the development of plastids.

4. Transport of related substances: Auxines are also structurally close to the spectrum of the substrates transported by the nuclear base transporters and could be similarly modified by the transporters. The entire spectrum of auxine operations can be influenced by an alteration in the transport of auxines.

5. Modification of the transport characteristics of transporters by mutagenesis. Broadening of the substrate spectrum, e.g. in the direction of the transport of alkaloids, which have hitherto not been recognized by the transporters, synthetic hormones, which are poorly transported, nuclear bases which are not transported

efficiently enough, as a springboard for the alteration of transport processes in transgenic plants.

Up till now homologues of almost all new plant transporters could be found in animal or human genomes (examples: glucose transporters of the plant family MST are related to the animal GLUT transporters at a sequence level, amino-acid transporters of the AAP family were later found in animals (VGAT, SN1); amino-acid transporters of the CAT family in animals and plants are related). On the basis of resemblance of biochemical properties, which were discovered for the animal/human transport of adenine and the competition by caffeine, it is postulated that a hitherto unidentified homologue of PUP is responsible for the nuclear base/ nucleoside and derivative transport in these systems. The expression of this system in yeast cells as an evidence of function enables first the description, then the identification of possible plant diseases provoked by defects in the systems, the identification of new structures leading to pharmaceuticals and the influencing of the transport of substances across the blood-brain barrier, since the adenine/caffeine transport occurs at this site

The following figures illustrate the invention.

Figure 1 shows a hydrophobicity analysis of the PUP nuclear base transporter protein after Kyte and Doolittle.

Figure 2 shows represented in the form of a diagram the results of an uptake experiment, in which the cytosine uptake rate of the yeast line MG877ura3⁻::pFL61-PUP1 and of the wild-type line Σ1278b (Dubois & Grenson, 1979, *Mol. Gen. Genet.* 175, 67-76) were measured at various pH levels. The substrate concentration amounted to 100µM.

Figure 3 shows represented in the form of a diagram the results of an uptake experiment in which the cytosine uptake of the yeast line MG877ura3⁻::pFL61-PUP1 was measured with and without the addition of glucose. The cells were washed twice with water and incubated at room temperature for 30 minutes before measurement of the uptake began. Five minutes after the measurements began a test supplementation of glucose to a final concentration of 1% was added.

Figure 4 shows an analysis of the substrate specificity of the PUP1 nuclear base transporter expressed in the yeast (black bar) compared to the yeast's own FCY-2 transporter (white bar).

Figure 5 shows represented in the form of a diagram the results of an uptake experiment in which the cytokinin uptake of the yeast line MG877ura3⁻::PUP1 was investigated by means of ^{3}H-labelled zeatine. The uptake was set temporarily at a concentration of 100 µM trans-zeatin. In this case PUP1 was expressed under control of the yeast ATPase promotor PMA1 in the vector pDR195 (Rentsch et al., 1995, *FEBS Lett.* 370, 264-368) in the mutant MG877ura3⁻::(FCY). The empty vector (FCY2pDR195) served as control.

Figure 6a shows a schematic representation of the plasmid p35S-PUP1, a derivative of the plasmid pBIN19 (Bevan, 1984, *Nucl. Acids Res.* 12, 8711-8721). A stands for a

fragment from the genome of the cauliflower mosaic virus, which carries the 35S promoter (nt 6909-7437). The promoter fragment was prepared as *EcoR1/Kpnl* fragment from the plasmid pDH51 (Pietrzak et al., *Nucl. Acids Res.* 14, 5857-5868). B stands for a *NotI/NotI* fragment of cDNA, flanked by a polylinker region from pT7T3/*NotI* with *Kpnl* and *Xbal* division sites with the coding region of the nuclear base transporter of *Arabidopsis thaliana* in sense orientation to fragment A. The arrow shown in fragment B indicates the reading direction of the cDNA. C stands for a polyadenylation signal of gene 3 of the T-DNA of plasmid pTiACH5 (Gielen et al., 1984 *EMBO J.* 3. 835-846). Nucleotides 11749 to 11939, which as *Pvul1/HindIII* fragment was isolated from the plasmid pAGV40 (Herrera-Estrella et al., 1983, *Nature* 303, 209-213) and cloned after addition of a *SphI* linker to the *PvuII* restriction site between the *SphI* and *HindIII* cleavage sites of the polylinker of pBIN19.

Figure 6b shows a schematic representation of the plasmid p35S-α-PUP1, a derivative of the plasmid pBIN19 (Bevan, 1984. *Nucl. Acids Res.* 12, 8711-8721). A and C in either case stand for the CaMV35S promoter and the polyadenylation signal of gene 3 of the T-DNA of the plasmid pTiACH5 (see figure 6a). B stands for a *SspI/HpaI* fragment of the cDNA with the coding region of the nuclear base transporter of *Arabidopsis thaliana* in anti-sense orientation to fragment A. The arrow shown in fragment B indicates the reading direction of the cDNA.

Figure 7 shows the measurement of one-way directed currents at a constant voltage of -80 mV expressing PUP1 in *Xenopus* oocytes. The one-way directed currents are measured as soon as adenine is added. The current strength is dependent on the concentration (bars indicate the current on the ordinate and the time on the abscissa).

Figure 8 shows (A) the increase of DM734-2841DpDR195 in 150µM adenosine (control) and (B) the increase of DM734-284DpDR195PuP1 in 150µM adenosine.

Figure 9 shows the growth of *Saccharomyces cerevisiae* MG877ura3⁻(K) and transformed clones with the construct pDR195PUP1 (1) or with the construct pDR195PUP2 on caffeine plates with various caffeine concentrations.

EXAMPLES

General Methods

a) Cloning procedure: For cloning in *E. coli* the vector pT7T3 18U (Pharmacia) and for transformation of yeasts the vector pFL61 (Minet & Lacroute , 1990, *Curr. Genet.* 18, 287-291) were inserted. For plant transformation the gene constructions in the binary vector pBinAR, a derivative of pBIN19 (Bevan, 1984, *Nucl. Acids Res.* 12, 8711-8721) were cloned .

b) Bacterial and yeast strains: For the pT7T3 18U- and pFL61 vectors as well as for the pBinAR constructs the *E. coli* strain DH5α was used. As initiating strain for the expression of the cDNA library in yeast the yeast strain MG887 (Dubois & Grenson, 1979, *Mol. Gen. Genet.* 175, 67-76) with the mutation *fcy2* was used, after a ura3 deficiency had been induced.

c) Transformation of *Agrobacterium tumefaciens*: DNA transfer in the Agrobacteria resulted through direct transformation by the method of Höfgren and Willmitzer (1988, *Nucl. Acids Res.* 16, 9877). The plasmid-DNA-transformed Agrobacteria were isolated by the method of Bimboim and Doly (1979, *Nucl. Acids Res.* 7, 1513-1523) and electrophoretically analysed following suitable restriction division.

d) Transformation of plants: The plant transfers can result from *Agrobacterium tumefaciens*-mediated (strain C58C1, pGV2260) gene transfer (Deblaere et al., 1985, *Nucl. Acids Res.* 13, 4777-4788). The transformation of *A. thaliana* is for example carried out by means of vacuum infiltration (modified after Bechtold et al. (1993) Comptes Rendus de l'Academie des Sciences Serie III, Sciences de la Vie 316: 1194-1199). Pots (diameter 10cm) are filled with earth and finally covered with a fly-net. On this net *A. thaliana* seeds are sown. Six to eight weeks after the sowing the plants are used for vacuum infiltration. For vacuum infiltration some of the corresponding *Agrobacterium* strains are cultivated as 2 x 1 litre cultures in YEB + antibiotic (50µg/ml kanamycin and 100µg/ml rifampicin) at 28°C. At OD_{600} the cells from 3000g are harvested and resuspended in 600ml infiltration medium (0.5 x MS medium (Sigma), 5% sucrose, 44µM benzylaminopurine. The bacterial suspension is filled into 250ml Weck tubes and placed in an exsiccator. The *A. thaliana* plants are immersed "head-up" in the bacterial suspension and then for 5 minutes vacuum is applied. After 3-4 weeks the seeds of these plants are harvested. For surface sterilization the seeds are shaken for 10 minutes in 4% sodium hypochlorite, 0.02% Triton, centrifuged out at 1500g, washed four times in sterile water and resuspended in 3ml 0.05% agarose per 5000 seeds. The seed-agarose solution is spread out on MSS medium (1 x MS, 1% sucrose, 0.8% agarose, 50µg/ml kanamycin, pH 5.8) (plates of 13.5cm diameter for 5000 seeds). To reduce the loss of moisture the plates are closed off with Parafilm®. The kanamycin-resistant plants are transferred to earth. Seeds from these plants are harvested and analysed.

e) Demonstration of nuclear base transporter activity: Demonstration of the activity can be carried out for example through uptake experiments with radioactive substrates (e.g. [^{14}C] adenine) in the *fcy2* yeast mutants (MG877ura3$^-$::pFL61-PUP1) transformed with the plant transporter gene under the control of the yeast promoter, in principle as described in Ninnemann et al., 1994 (*EMBO J.* 15, 3464-3471).Alternatively other expression systems may be introduced, e.g. *Xenopus* oocytes (Boorer et al., 1996, *J. Biol. Chem.* 271, 2213-2220), with the employment of electrophysiological measurement methods.

Example 1: cloning of the PUP1 nuclear base transporter gene from *Arabidopsis thaliana*

The cloning of the PUP1 nuclear base transporter was accomplished by complementation of the yeast strain MG887ura3$^-$(*fyc2*) (this work; preliminary step MG887 Grenson 1969, *Eur. J. Biochem.* 11, 249-260) with a cDNA gene bank from *Arabidopsis thaliana* and the selection of nuclear base transporter positive cells. The *fyc2* yeast mutant cannot grow in media with adenine or cytosine as the only source of

nitrogen (Grenson , 1969, *Eur. J. Biochem.* 11, 249-260; Polak & Grenson, 1973, *Eur. J. Biochem.* 32, 276-282). For the introduction of an auxotrophy marker (ura3$^-$) the nuclear uptake-deficient mutant MG887 (Dubois & Grenson, 1979, *Mol. Gen. Genet.* 175, 67-76) was transformed with a fragment of the URA3 gene, which carries an internal deletion. Through selection at the toxic preliminary step 5-fluoro-orotate a URA-deficient mutant MG887ura3$^-$ could be isolated.

For complementation of the nuclear base transport mutation of the yeast strain MG887ura3$^-$ cloned cDNA from young embryos of *Arabidopsis thaliana* (at the two-leaf stage) are used (Minet et al., 1992, *Plant J.* 2, 417-722). About 1µg of the vector with a cDNA insertion was transformed in the yeast strain MG887ura3$^-$ according to the method of Dohmen et al. (1991, *Yeast* 7, 691-692). Yeast transformants which could grow in minimal medium with 1mM adenine as the only source of nitrogen were propagated. From these clones plasmid DNA was isolated by standard means. The strain MG887ura3$^-$ was once more transformed with the plasmid isolated. In this way a plasmid pFL61-PUP1 which can complement the *fcy2* mutation was obtained. This plasmid has an insertion of 1.2 kilobases containing the PUP1 nuclear base transporter gene.

The yeast strain MG887ura3$^-$::pFL61-PUP1 obtained by transformation of MG887ura3$^-$ with the plasmid pFL61-PUP1 is used for uptake studies with adenine or nuclear bases. Through gene engineering alteration of the coding region of the nuclear base transporter gene PUP1 according to standard procedures (cf. Sambrook et al., Molecular cloning: a laboratory manual, 2nd edn., Cold Spring Harbor Laboratory Press, NY, USA) its specificity or the characteristics of the transport mechanism may be changed. The strain MG887ura3$^-$::pFL61-PUP1 is directly suitable for the investigation of inhibitors or promoters of nuclear base transport.

The PUP1 protein expressed by the yeast strain MG887ura3$^-$::pFL61-PUP1 was subjected to hydrophobocity analysis by the method of Kyte and Doolittle. As can be seen from Fig. 1, the PUP1 protein displays 9-12 strongly hydrophobic regions (positive values on the Y axis) which are long enough in each instance to stretch once across the membrane. The first hydrophobic region is not conserved in all PUP proteins.

Sequence analysis of the cDNA insertion of the pFL61-PUP1 plasmid

The plasmid pFL61-PUP was isolated from the yeast strain MG887ura3$^-$::pFL61-PUP1 and with the aid of synthetic oligonucleotides the insertion was sequenced by the method of Sanger et al.(1977, *Proc. Natl. Acad. Sci USA* 74, 5463-5467). The coding sequence of the PUP1 gene is reproduced in SEQ ID NO 1 and that of the protein sequence derived from it in SEQ ID NO 8.

Example 2: Uptake studies on the yeast strain MG887ura3$^-$pFL61-PUP1 with ^{14}C-labelled cytosine and ^{3}H-labelled cytokinins.

For measuring the rate of uptake the yeast strains MG887ura3$^-$::pFL61, MG887ura3$^-$::pFL61-PUP1 and their original strain Σ1278b (Dubois & Grenson, 1979, *Mol. Gen. Genet.* 175, 67-76) were cultured in complete medium (YPD) without uracil and with

2% glucose as carbon source up to an OD_{600} of 0.6 at 28°C. The cells were harvested at 3000g, washed twice with water and brought to an OD600 of 12 with sodium phosphate buffer (100mM, pH 4.5), 1% glucose. The cell suspension was stored in 100µl portions on ice up to the beginning of the uptake measurements. Before the start of the uptake measurements the cells were pre-incubated at 30°C for 2 minutes. The reaction was then started, by which 100µl of radioactively labelled substrate solution was added to 100µl cell suspension. The reaction mixture was incubated at 30°C and after 30, 60, 120 and 180 seconds in each case 50µl of suspension was withdrawn and placed in 4ml of ice-cold water (in measurements over 180 seconds more cell suspension and substrate solution were correspondingly added). The cells were absorbed on to glass fibre filters and washed with 4ml of ice-cold water. The radioactivity on the filters was finally ascertained in a liquid scintillation counter.

Substrate solution: For the substrate solution what is concerned is a 100mM sodium phosphate buffer pH 4.5 with 1% glucose and 9.25 Bq/µl of radioactively labelled substrate (25-100 µM [^{14}C] adenine or [^{14}C] cytosine). In addition the substrate solution contains the unlabelled substrate, possible inhibitors and competitors in double end-concentration. For estimation of the optimum pH the pH value of the sodium phosphate buffer is also changed. When the influence of glucose on the uptake rate is measured neither the cell suspension nor the substrate solution contains glucose. Glucose is only added after starting the uptake measurements at the commencement of measuring at an end-concentration of 1%. Furthermore it could be shown that PUP2 was also in a position to transport adenine.

In Table 1 the uptake of radioactively labelled adenine and cytosine mediated by the PUP1 nuclear base transporter is indicated by the yeast strain MG887ura3$^-$::pFL61-PUP1. For calculation of the intracellular concentration the cell volume is estimated as four times the dry weight (Ninnemann et al., 1994, *EMBO J.* 15, 3464-3471). The uptake occurs against a concentration gradient.

Table 1

	Initial concentration Medium	*End conc. Medium*	*End conc. Cells*	*Enrichment factor*
Adenine	200µM	155µM	2350µM	15
Cytosine	200µM	145µM	2660µM	18

The influence of various inhibitors on the adenine or cytosine uptake mediated by the PUP1 nuclear base transporter in the yeast strain MG887ura3$^-$::pFL61-PUP1 is indicated in Table 2. The inhibitors were added to the cells five minutes before beginning the measurements. The protonophores carbonyl-cyanide-m-chlorphenylhydrazone (CCCP) and 2,4-dinitrophenol (2,4-DNP) and the H^+/ATPase inhibitor diethylstilboestrol block the uptake. This may be taken as a clear indication of a secondarily active proton-associated uptake mechanism

Table 2

Inhibitor	*Relative adenine uptake*	*Relative cytosine uptake*

	[%]	[%]
without inhibitor	100	100
100µM diethylstilboestrol	4	8
100µM DCCD	47	55
100µM CCCP	13	20
100µM 2,4-DNP	54	58
10µg/ml cycloheximide	95	97

Figure 2 shows that the cytosine uptake into the yeast strain MG887ura3$^-$::pFL61-PUP1 mediated by the PUP1 nuclear base transporter is dependent on the pH level. Similar results were attained for the adenine uptake (data not shown). As shown in Fig. 3, the cytosine uptake into the yeast strain MG887ura3$^-$::pFL61-PUP1 mediated by the PUP1 nuclear base transporter is glucose-dependent. Similar results were obtained for the adenine uptake (data not shown). The increase of activity on the addition of glucose may be taken as an indication of energy dependence. This as well as the observed increase of activity with a decline in the pH level points towards a secondarily active proton-associated uptake mechanism.

For investigating the substrate specificity of the PUP1 nuclear base transporter expressed in yeast in comparison to the yeast's own FCY2 transporter, competition experiments with non-radioactive substrates were carried out. The results of these experiments are shown in Fig. 4.The uptake of radioactively labelled adenine was measured set at 100% (corresponding to 1.7 or 0.9 nmol.min^{-1}.mg^{-1} dry weight). The measurements were carried out at a substrate concentration of 25µM. The competitors were added in 10 times molar excess.

For the analysis of competitive inhibition by the cytokinins kinetin and zeatin of adenine transport mediated by the PUP1 nuclear base transporter uptake investigations were carried out with different concentrations of radioactively labelled adenine as well as various competitor concentrations. From Lineweaver/Burk calculations the inhibitor constants Ki were determined (35µM for kinetin and 30µM for zeatin).

Direct uptake experiments in PUP1-expressing yeast cells with ^{3}H-labelled cytokinins (zeatine and 2-isopentenyl adenine) show that PUP1 can also transport cytokinins (Figure 5). The uptake experiments were carried out with ^{3}H-labelled zeatine in MG887ura3$^-$ as for adenine. To start the reaction 87.3Bq/µl of the radioactive substrate was added to a total concentration of 100µM trans-zeatine. The results of three independent experiments showed a 70 times increased uptake of radioactively labelled trans-zeatine for the PUP1-expressing yeast clone as against the control strain transformed with the control plasmid pDR195. This shows that PUP1 mediates the transport of trans-zeatine.

Example 3. Transformation of plants with constructions for over-expression or antisense repression of a nuclear base transporter gene

The introduction of a nucleic acid coding for a plant nuclear base transporter and the over-expression or the antisense repression of a nuclear base transporter gene for the

alteration of the transport of nuclear bases and their derivatives is described here with *Arabidopsis thaliana* as example. Its use is nevertheless not confined to this species.

a) Over-expression: The 1.2kb *NotI* fragment from the plasmid pFLP-PUP1, which contains as an insertion the cDNA for a nuclear base transporter of *Arabidopsis thaliana*, was cloned in the vector pT7T3 18U/ *NotI* excised with *NotI*. The vector pT7T3 18U/*NotI* was created because in the *SmaI* excision site of pT7T3 18U a *NotI* linker was included. The orientation of the fragment was checked by restriction splitting. The vector includes a *KpnI* and an *XbaI* excision site in the multiple cloning site, so that the PUP1 cDNA from this vector can be isolated as a *KpnI-XbaI* fragment. This 1.2 kb fragment was cloned in the *KpnI-XbaI* excised vector pBinAR, a derivative of pBIN19 (Bevan, 1984, *Nucl. Acids Res.* 12, 8711-8720). The resulting plasmid was named p35S-PUP1.

b) Anti-sense repression: from the plasmid pFL61-PUP1 a 1.1kb-sized HpaI-SspI-PUP1 fragment was isolated and cloned in anti-sense orientation in the SmaI incision site of pBinAR. The orientation of the fragment was checked by restriction splitting. The resulting plasmid was named p35-α-PUP1.

The PUP1-cDNA fragments bear the designation "B" in Figures 6a and 6b. Whatever the case, whether B was incorporated in sense orientation to the CaMV-35S promoter of pBinAR or not, the resulting plasmid carries the designation p35S-PUP1 or p35S-α-PUP1. Between their *EcoRI* and *KpnI* excision sites a fragment from the genome of the cauliflower mosaic virus which carries the 35S promoter (nt 6909-7437) is inserted. The promoter fragment is prepared as an *EcoRI/KpnI* fragment from the plasmid pDH51 (Pietrzak et al., *Nucl. Acids Res.* 14, 5857-5868). In the plasmid map the promoter fragment bears the designation "A". Between the *SphI* and *HindIII* excision sites of pBinAR is inserted in addition the polyadenylation signal of gene 3 of the T-DNA of the plasmid pTiACHS (Gielen et al., *EMBO J.* 3, 835-846). For this purpose a *PvuII/HindIII* fragment (nt11749-11939) from the plasmid pAGV 40 (Herrera-Estrella et al., 1983, *Nature* 303, 209-2139) had a *SphI* linker attached at the *PvuII* excision site. The polyadenylation signal carries the designation "C" in the plasmid map.

Following transformation of agrobacteria with the plasmids p35S-PUP1 and p35S-α-PUP1 these were put into the vacuum filtration of *Arabidopsis thaliana*.

Ten independently obtained transformants for both constructs, in which the presence of the intact, not rearranged, chimerical genes had been demonstrated with the aid of "Southern Blot" analyses, were investigated in connection with the changes in nuclear base transport.

Example 4: Expression of the PUP1 gene in *Xenopus* oocytes.

The cDNA for PUP1 was excised with *NotI* and cloned in an oocyte expression vector containing untranslated 5' and 3' regions of the β-globin from *Xenopus*. The cDNA was then replicated by means of PCR. SP6 (from the oocyte vector) was used as upstream primer, as downstream primer a primer was used which demolishes the

STOP codon of PUP1 and simultaneously inserts a *NotI* excision site. This PCR fragment was excised by *NotI* and *HindIII* and ligated into an oocyte expression vector which in addition contains behind the *NotI* excision site the gene for GFP ("green fluorescent protein" from the jellyfish). In that way an open reading-frame is constructed for a fusion protein from PUP1 and GFP. By insertion of the *NotI* excision site a linker with three base pairs coding for alanine is constructed. The plasmid was linearized with *MluI*, and RNA was transcribed *in vitro* by means of SP6 polymerase and absorbed into water to about 1 ng/nl. Each oocyte (ripening stage 5 to 6) was injected with 50nl. Following twenty days' incubation at 16° oocytes were placed for measurement in an appropriate measurement chamber and rinsed over with various solutions. The solutions contained: 100mM N-methyl-D-glutamine chloride, 2mM calcium chloride, 5mM MES, pH 5.0 or 7.3, and corresponding concentrations of adenine.

For voltage measurement a glass electrode filled with 3M KCl was stuck into the oocyte. With a Dagan amplifier the potential was measured relative to the reference electrode. This was connected with the bath solution via an agar bridge. In solutions without adenine at pH 7.3 oocytes had a typical resting potential of about -30mV (see figure 7). Adenine in the bath solution provokes depolarization of the oocyte. This indicates that PUP1 transports adenine even in oocytes.

For measurement of current the oocytes were held at a voltage of -80mV. Adenine in the bath solution induces a current in one direction. Since adenine itself carries no charge, that indicates a cotransport with charged ions, e.g. protons.

Example 5: PUP1-mediated transport of adenosine in yeast

The *Saccharomyces cerevisiae* strain DM734-284D *(genotype: ade8-18, ade2-1, arg4-16, leu2-27, trp1-1, lys2, ura3;* Yeast Genetic Stock Center) was used for growth studies. This yeast strain owing to mutations at the gene loci ade 8-18 and ade 2-1 is not itself in a position to synthesize adenine. Hence this strain needs adenine from the external medium to be able to grow. This adenine is taken up via the purine/cytosine transporter FCY2 of the yeast. The DM734-284D strain is however still in a position to grow in medium containing adenosine instead of adenine, provided adenosine transport in the yeast cells is mediated by an adenosine transporter inserted by genetic engineering. *Saccharomyces cerevisiae* itself does not possess an adenosine transporter of such a kind. Hence the strain DM734-284D is suitable as a complementation system for the isolation of adenosine transporters from various organisms.

In order to check whether PUP1 mediates the transport of adenosine, the strain DM734-284D was transformed according to the standard procedure for yeast transformation (Dohmen et al., 1991, *Yeast* 7, 691-692) with the constructs pDR195PUP1 and pDR195 as controls (Rentsch et al., 1995, *FEBS Lett.* 370, 264-238). For final checking of the transformation the early stages of transformation were plated on minimal medium containing 150µM adenine and the amino acids arginine, leucine, tryptophane (each 60mg/l) and lysine (70mg/l) necessary for growth. Uracil served as transformation marker. Three independent positive clones of the control and the PUP1-expressing clone were plated on minimal medium containing 150µM

adenosine instead of 150µM adenine. Definite growth was shown after 3 days by the PUP1-expressing clone, while the clone transformed with the vector pDR195 showed no growth (see Figure 8). This indicates that PUP1 mediates the transport of the nucleoside and adenine-derivative adenosine.

Example 6: PUP1-mediated transport of caffeine in yeast

In order to check whether PUP1 mediates the transport of caffeine, the sensitivity of the *Saccharomyces cerevisiae* strain MG877ura3⁻ to caffeine-containing minimal medium was investigated. At certain concentrations caffeine is toxic to yeast (Bard et al., *J. Bacteriol.* 141, 999-1002), leading to slower growth or death of the yeast. If PUP1 mediates the transport of caffeine it may be assumed that a yeast strain expressing this protein possesses a greater sensitivity to caffeine, manifested as reduced growth, than the corresponding control strain.

For this test various caffeine concentrations between 0 and 1.5% were put into minimal medium. Yeast clones grown in liquid minimal medium for 16 hours were spread out on the corresponding plates and incubated for 6 days at 28°C. It was indicated by this that PUP1-expressing yeasts displayed distinctly slowed-down growth compared to the control strain MG877ura3⁻ and the PUP2-expressing strain from the concentration of 0.2% caffeine on (see Figure 9). This must be ascribed to the increased uptake of toxic caffeine and shows that PUP1 mediates the transport of caffeine.